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Published in:
GigaScience

DOI:
[10.1093/gigascience/gix073](https://doi.org/10.1093/gigascience/gix073)

Publication date:
2017

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Mitterboeck, T. F., Liu, S., Adamowicz, S. J., Fu, J., Zhang, R., Song, W., Meusemann, K., & Zhou, X. (2017). Positive and relaxed selection associated with flight evolution and loss in insect transcriptomes. *GigaScience*, 6(10), 1-14. <https://doi.org/10.1093/gigascience/gix073>

RESEARCH

Positive and relaxed selection associated with flight evolution and loss in insect transcriptomes

T. Fatima Mitterboeck^{1,2,†}, Shanlin Liu^{3,4,†}, Sarah J. Adamowicz^{1,2}, Jinzhong Fu¹, Rui Zhang³, Wenhui Song³, Karen Meusemann^{5,6,7} and Xin Zhou^{8,9,*}

¹Department of Integrative Biology, University of Guelph, 50 Stone Road East, Guelph, ON, N1G 2W1 Canada,

²Biodiversity Institute of Ontario, University of Guelph, 50 Stone Road East, Guelph, ON, N1G 2W1 Canada,

³BGI-Shenzhen, Beishan Industrial Zone, Yantian District, Shenzhen, Guangdong Province, 518083 China,

⁴Centre for GeoGenetics, Natural History Museum of Denmark, University of Copenhagen, Øster Voldgade 5–7, 1350 Copenhagen, Denmark, ⁵University of Freiburg, Department for Biology I (Zoology), Evolutionary Biology and Ecology, Hauptstr. 1, D-79104 Freiburg, Germany, ⁶Center for Molecular Biodiversity Research, Zoological Research Museum Alexander Koenig, Adenauerallee 160, 53113 Bonn, Germany, ⁷Australian National Insect Collection CSIRO, Natl Collections & Marine Infrastructure, Clunies Ross Street, ACTON, 2601 ACT, Canberra, Australia, ⁸Beijing Advanced Innovation Center for Food Nutrition and Human Health, China Agricultural University, 2 West Yuanmingyuan Rd., Haidian District, Beijing 100193, China and ⁹College of Plant Protection, China Agricultural University, 2 West Yuanmingyuan Rd., Haidian District, Beijing 100193, China

*Correspondence address. Xin Zhou, Beijing Advanced Innovation Center for Food Nutrition and Human Health & College of Plant Protection, China Agricultural University, 2 West Yuanmingyuan Rd., Haidian District, Beijing 100193, China. Tel: +86 010-62732829; Fax: +86 010-62731209;

E-mail: xinzhou@cau.edu.cn

[†]Shared first authorship

Abstract

The evolution of powered flight is a major innovation that has facilitated the success of insects. Previously, studies of birds, bats, and insects have detected molecular signatures of differing selection regimes in energy-related genes associated with flight evolution and/or loss. Here, using DNA sequences from more than 1000 nuclear and mitochondrial protein-coding genes obtained from insect transcriptomes, we conduct a broader exploration of which gene categories display positive and relaxed selection at the origin of flight as well as with multiple independent losses of flight. We detected a number of categories of nuclear genes more often under positive selection in the lineage leading to the winged insects (Pterygota), related to catabolic processes such as proteases, as well as splicing-related genes. Flight loss was associated with relaxed selection signatures in splicing genes, mirroring the results for flight evolution. Similar to previous studies of flight loss in various animal taxa, we observed consistently higher nonsynonymous-to-synonymous substitution ratios in mitochondrial genes of flightless lineages, indicative of relaxed selection in energy-related genes. While oxidative phosphorylation genes were not detected as being under selection with the origin of flight specifically, they were most often detected as being

Received: 7 March 2017; Revised: 14 July 2017; Accepted: 1 August 2017

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under positive selection in holometabolous (complete metamorphosis) insects as compared with other insect lineages. This study supports some convergence in gene-specific selection pressures associated with flight ability, and the exploratory analysis provided some new insights into gene categories potentially associated with the gain and loss of flight in insects.

Keywords: Insect transcriptomes; flight; flight loss; positive selection; 1KITE project; molecular evolution

Background

The evolution of active flight in insects has most likely had a positive impact on the species diversity of this group [1]. Flight, having arisen multiple times in animals, arose earliest in insects approximately 400 million years ago and characterizes the clade Pterygota [2]. The evolution of key traits at the origin of Pterygota is not well understood; wings may have originated from the modification of gills, extensions of the body wall, or both [3–5]. By increasing dispersal ability, flight facilitates the finding of food and mates as well as the avoidance of unfavourable habitats or predators [6]. In addition to the evolution of flight, pterygote insects evolved incomplete metamorphosis, which involves egg, nymph, and adult stages. These transitions paved the way for later innovations within Pterygota, such as wing folding and complete metamorphosis as occurring in holometabolous insects (i.e., egg, larval, pupal, and adult stages), which are additionally implicated in the evolutionary success of insects [1]. Despite the advantages associated with active flight, it has been estimated that flight has been lost thousands of times within pterygotes [7], such as in lineages representing fleas, snowflies, and stick insects [8].

Powered flight is a highly energetically costly activity in animals, including in birds and bats [9, 10]. Flying insects use up to 50 [11] or 100 times [12] more energy when flying than at rest. The oxidative phosphorylation (OXPHOS) pathway in the mitochondrion provides 95% of the energy required for eukaryotic cells [13]. Therefore, the 13 mitochondrial protein-coding OXPHOS genes, the 78 nuclear OXPHOS genes (number present in *Drosophila*) [14], and the hundreds of additional nuclear-encoded genes that function in the mitochondria (postulated in *Drosophila melanogaster*) [15] are likely important in the evolution of traits that require large amounts of energy [10], such as large brain:body size ratios [16]. Genes involved in energy production, such as mitochondrial protein-coding genes, were observed to bear signatures of positive selection with the evolution of flight in animals, or conversely under relaxed selection with flight loss [9, 10, 17, 18]. However, the association between genes of other functional groups and flight evolution in insects has not been investigated, with most previous studies focused on mitochondrial energy-related genes *a priori* [18].

Developmental and gene expression studies have investigated genes relevant to wings or flight ability. Genes important for the physical development of wings have been identified, including the protein-coding genes *wingless*, *apterous*, *vestigial*, *nubbin*, *nub* [19], and *vein* [20]. Genes differentially expressed in flying and non-flying morphs within certain insect species have also been identified. Genes more highly expressed in flying morphs include (i) those involved in energy production, such as genes that function in the mitochondria [21, 22] and the nuclear gene *Isocitrate dehydrogenase* (IDH), which is important in the citric acid cycle [22]; (ii) those involved with lipid metabolism [21]; and (iii) the *flightin* gene [21–23], which is important for indirect flight muscle function [24]. Genes more highly expressed in flightless morphs include those related to sugar metabolism [21], such as *trehalase* (involved in conversion of trehalose to glucose) [22] and *seryl-tRNA synthetase* (involved in tRNA metabolic

processes) [21]. Functions of genes observed to be differentially expressed between flying insect individuals with higher vs lower flight metabolic rates include ribosome/RNA processing [25], while genes exhibiting differences between long- vs short-distance flight migrators include those involved in lipid mobilization and flight muscle structure [26]. Additionally, particular splice forms of certain genes such as encoding glycerol-3-phosphate dehydrogenase (functions in the glycolytic pathway to produce ATP) appear necessary for flight [27], with the relative abundance of various splice variants affecting the power output of flight muscles, as shown in a dragonfly species [28]. Similar categories of genes could be under differential selection pressures associated with the evolutionary gain and loss of flight; however, this has not yet been tested directly with selection analysis.

We explore what types of protein-coding genes have experienced differing selective pressures associated with the evolution and loss of flight using DNA sequences from a total of 1476 nuclear single-copy orthologous protein-coding genes and 13 mitochondrial protein-coding genes obtained from transcriptomes. First, we test for evidence of positive selection during the time when flight originated. Second, we test for positive and relaxed selection among multiple evolutionary losses of flight, which provide more recent and naturally replicated evidence for genes potentially associated with the evolution and maintenance of flight. In addition to using multiple evolutionary shifts in a biological or ecological trait to identify common genetic trends associated with that shift (e.g., [9, 29]), we additionally use the reverse direction event to serve as comparison with the sole case of flight gain in hexapods. Third, to examine further the relationship between energy-related genes and flight, we test for positive selection in available nuclear OXPHOS and mitochondrial OXPHOS genes throughout the major lineages of hexapods.

Data Description

The nuclear genetic data used in this study consist of transcriptome-derived DNA sequences obtained as part of the 1000 Insect Transcriptome Evolution (1KITE) project [30] and additional hexapod genomes, as is presented in Misof et al. [2]. We utilized the current assembly version 2 (strict assembly followed by check for cross-contamination, described in Mayer et al. [31]) of transcript data of 101 species (NCBI accession PRJNA183205, individual accessions provided in the Supplementary Data, Table S1) [2, 32] and assigned transcripts to 1476 single-copy nuclear orthologous genes included in the ortholog set published by Misof et al. [2]. We additionally included the 12 reference species with an official gene set available and used by Misof et al. [2] to infer orthology; thus, data for 113 species were available in total. Orthology assignment of transcripts, alignment, outlier check, alignment refinement, and generation of nucleotide alignments followed the guidelines described in Misof et al. [2] with some modifications (see the “Methods” section). Sequences for the 13 mitochondrial protein-coding genes were obtained from the associated mitochondrial transcriptome sequencing project of BGI, with some substitution of sequences from

mitochondrial genomes published on NCBI to increase completeness (Table S13) (species and sources of data provided in Mitterboeck et al. [32]). Mitochondrial sequences were aligned with EMBL-EBI Clustal Omega (Clustal Omega, [RRID:SCR.001591](#)) [33] and Pal2Nal [34]. Guidance [35] was applied to mask sequence regions that were unreliably aligned. The phylogenetic tree topology used here for selection tests was obtained from Misof et al. [2]. The data sets supporting the results of this article are available in the GigaDB repository [32].

Analyses

Positive selection associated with the origin of flight

Tests of positive selection were performed for each lineage of interest via branch site models, which estimate dN/dS ratios at each codon site and between branches such that positive selection is detected in the lineage of interest if a subset of codon sites have dN/dS ratios greater than 1, while the other lineages have ratios of less than 1 or equal to 1, indicating purifying selection or neutral evolution, respectively. Out of 954 nuclear genes tested in the lineage leading to the pterygote insects (“P” in Fig. 1), 126 (13%) were detected to be under positive selection; 39 of these were uniquely detected to be under positive selection in branch “P” and not detected in either branch “U” (upstream) or “D” (downstream). The 39 unique candidate genes over-represented gene ontology categories related to “spliceosome,” “protein binding,” “protease,” and “RNA catabolic process” (Table 1). The candidate gene list included *frayed* (*fray*) and NADH dehydrogenase (ubiquinone) 23 kDa subunit (ND-23), related to wing development and the mitochondrial respiratory chain, respectively, but such functional categories did not contain an over-representation of genes exhibiting evidence of positive selection. When grouping multiple gene ontology terms of potential interest related to wing or mitochondrion/ATP-binding/OXPHOS-related functions, neither of these groupings was significantly over- or under-represented by the 39 candidate genes as compared to the non-candidate genes (wing: 2.6% in candidate list of genes displaying signature of positive selection vs 3.0% in non-candidate list, $P_{\text{Fisher's exact (1-tailed)}} = 0.69$; mitochondrion-related: 15.8% vs 17.4%, $P = 0.67$; only over-representation P values shown). Out of 13 nuclear OXPHOS genes available in the background gene set of 954, only 1 was in the candidate list (2.6% vs 1.3%, $P = 0.42$). None of the 13 mitochondrial genes was detected to be under positive selection in the “P” lineage after Benjamini-Hochberg correction. Gene names and descriptions for candidate and background genes for all analyses are provided in [32] Table S15.

Positive selection associated with flight loss

Eleven lineages (Fig. 1) representing flight losses had between 0.8% and 53.7% of genes exhibiting positive selection, with a median of 2.4%. After considering the counts of genes detected under positive selection in the selected related flying lineages, 21 genes were still commonly (in 3 or more lineages) under positive selection in the flightless lineages. These genes over-represented the gene ontology categories of “coiled coil” (a protein structural motif), “nucleus,” and “dendrite morphogenesis” (Table 2). When considering only the 8 fully flightless lineages (excluding female flightless lineages) and 7 selected related flying lineages, the gene ontology categories for the candidate genes were similar; they included the 3 listed above, plus “DNA binding,” “cytosol,” and “developmental protein,”

as well as process categories additionally including “protein methylation” (Table S11) [32]. These 17 genes did not over- or under-represent gene category descriptions relating to wings or mitochondrion/ATP-binding/OXPHOS-related functions, with the wing-related genes being *absent*, *small*, or *homeotic discs 2* (*ash2*) and *no ocelli* (*noc*; 11.8% in candidate list vs 3.1% in non-candidate, $P = 0.099$) and 2 ATP-linked genes including *gluon* (*glu*; 11.8% vs 17.2%, $P = 0.82$). No nuclear OXPHOS genes were present in the 17-gene candidate list among the 13 nuclear OXPHOS genes tested ($P = 1.0$).

Relaxed selection associated with flight loss

Postulated relaxed selection was detected by increased dN/dS ratios across the fully flightless vs flight-capable branches of the tree (i.e., pooling branches by flight state) calculated for the entire length of each gene tested, as opposed to positive selection, which was detected using branch site models (accounting for dN/dS ratios at each site) on individual lineages of interest. Fifty-six out of 1285 nuclear genes tested show significantly higher ($P < 0.05$) dN/dS ratios in the flightless pterygote lineages than in related flying lineages (red vs blue lineages in Fig. 1). None of the 56 candidate genes overlapped with the 17 genes detected as candidates in the positive selection analysis of fully flightless lineages. The main gene ontology (GO) categories were related to “spliceosome,” while processes were “RNA localization,” “negative regulation of apoptotic processes,” and “extracellular transport” (Table 3). The candidate gene descriptions contained wing-related functions in genes that included *tankyrase* (*Tnks*), and wing- and ATP-binding-related functions in the gene *tricornered* (*trc*). Neither the wing-related nor mitochondrion/ATP-binding/OXPHOS-related groupings were over- or under-represented compared to the non-candidate genes (wing: 3.7 vs 3.4%, $P = 0.56$; mitochondrion: 13.0% vs 17.9%, $P = 0.87$). None of the nuclear OXPHOS genes were present in the candidate gene set out of 14 nuclear OXPHOS genes tested ($P = 1.0$). Only 2 nuclear OXPHOS genes had higher dN/dS ratios in flightless lineages, with 12 showing higher dN/dS ratios in flying lineages (12 out of 14, $P_{\text{binomial}} = 0.013$), and 4 of those exhibited a significant difference (Table S9) [32]. The *myosin binding subunit* (*Mbs*) gene ($P = 1.0 \times 10^{-16}$) and *IDH* gene ($P = 0.050$) showed higher dN/dS ratios in flying than flightless lineages. The mitochondrial genes showed significantly higher dN/dS ratios in the flightless pterygote lineages, which here included both sexes-flightless and female-flightless lineages, than in the related flying lineages (Fig. 2). Eleven out of 13 mitochondrial OXPHOS genes ($P_{\text{binomial}} = 0.023$), and all 5 of the genes exhibiting a significant difference had higher dN/dS ratios in the flightless lineage than in the related flying lineage (P values given in Table S10) [32].

Overlap between positive and relaxed selection results

Three biological process categories overlapped between the positive selection analyses from the Pterygota lineage (39 candidate genes) and the relaxed selection analyses in flightless vs flying lineages (56 candidate genes): “mRNA splicing, via spliceosome,” “catalytic step 2 spliceosome,” and “precatalytic spliceosome.” Two genes overlapped between these candidate lists of genes under positive or relaxed selection, out of 933 genes in common between the 2 sets of tests: *hephaestus* (*heph*) and *Ribosomal protein L13A* (*RpL13A*), together belonging to the DAVID functional annotation term “mRNA binding” ($P_{\text{DAVID}} = 0.035$) (Table S11) [32].

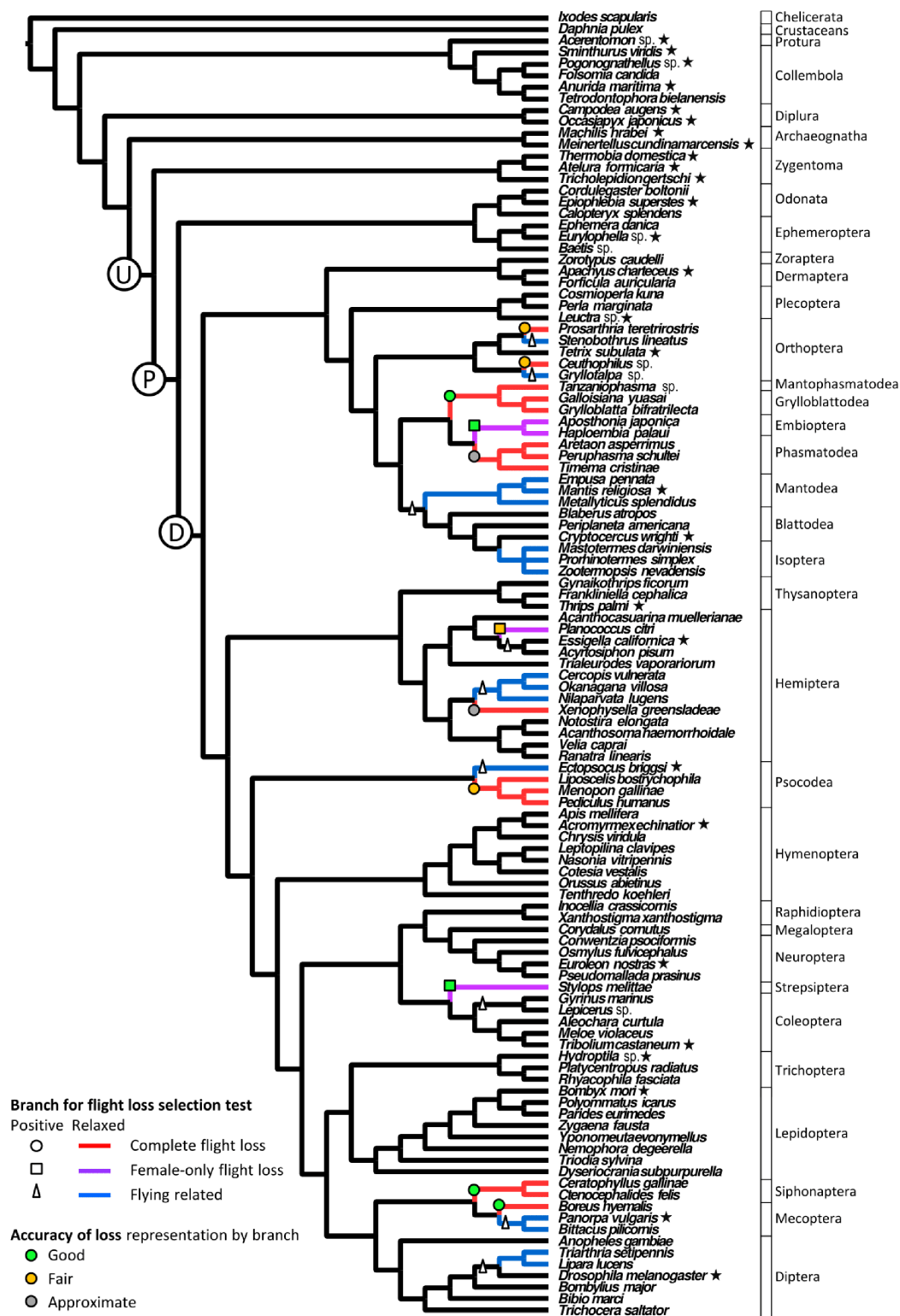


Figure 1: Tree topology and species used in analyses of nuclear genes. Species names followed by a star indicate those species used in positive selection analysis associated with the origin of Pterygota (branch “P”) and other lineages for comparison (branches “U” and “D”). Circles or squares on the branches indicate each of the 11 lineages that were used in positive selection analysis of flight loss, with circles indicating full flight loss and squares indicating female-only flight loss. Triangles indicate related flight-capable branches used for comparison with the lineages representing a loss of flight in positive selection analysis. Note that sub-trees were used for the positive selection tests, and so not all species shown here were included. The colour of the circles or squares indicates the estimated degree of accuracy in the phylogenetic mapping of the flight loss, given the available taxonomic sampling (green = good, orange = fair, grey = approximate). Red lineages (fully flightless) were compared with blue lineages (related flying) in the nuclear gene analyses of relaxed selection (dN/dS ratios) associated with flightlessness, with all other lineages used for a background rate. A similar (smaller) tree was used for mitochondrial gene analyses of relaxed selection where both red (fully flightless) and purple (female-only flightless) lineages were compared with blue (related flying) lineages, with other lineages representing the background rate.

Table 1: Positively selected genes in the lineage ("P") leading to Pterygota as over-represented in (A) GO categories from DAVID analysis and (B) Biological Process categories from PANTHER analysis

(A) DAVID GO results					
GO term	914 total genes # in category	38 positively selected genes		P value	Fold enrichment
		Expected #	Observed #		
Precatalytic spliceosome	34	1.4	7	0.00084	5.0
mRNA splicing, via spliceosome	44	1.8	8	0.00087	4.4
Catalytic step 2 spliceosome	30	1.2	6	0.0032	4.8
Protein binding	81	3.4	8	0.035	2.4
Protease	19	0.8	4	0.038	5.1
mRNA processing	9	0.4	3	0.044	8.0
(B) PANTHER Biological Process results					
PANTHER GO-Slim Biological Process term	894 total genes # in category	35 positively selected genes		P value	Fold enrichment
		Expected #	Observed #		
RNA catabolic process (GO:0 006401)	9	0.4	2	0.048	5.7

Terms are for positively selected genes uniquely detected in the "P" lineage and not in 2 control lineages tested ("U" and "D"). Categories with $P < 0.05$ are shown; full results are given in Table S11 [32]; 954 background genes were mapped to (A) 914 IDs and (B) 894 IDs; 39 unique candidate genes were mapped to (A) 38 IDs and (B) 35 IDs. Statistical over-representation is tested by modified Fisher's exact tests in DAVID and binomial statistics in PANTHER, with raw P values provided here.

Table 2: Genes detected to be under positive selection in 3 or more lineages with flight loss as over-represented in (A) GO categories from DAVID analysis and (B) Biological Process categories from PANTHER analysis

(A) DAVID GO results					
GO term	1229 total genes # in category	21 candidate positively selected genes		P value	Fold enrichment
		Expected #	Observed #		
Coiled coil	223	3.8	9	0.018	2.4
Nucleus	269	4.6	9	0.048	2.0
Dendrite morphogenesis	21	0.4	3	0.050	8.4
(B) PANTHER Biological Process results					
PANTHER GO-Slim Biological Process term	1207 total genes # in category	21 positively selected genes		P value	Fold enrichment
		Expected #	Observed #		
Cellular component organization	113	2.0	5	0.041	2.5
Organelle organization	64	1.1	4	0.0229	3.6
Chromatin organization	18	0.3	2	0.0387	6.4

Counts of positively selected genes in related flying lineages were removed from counts in flightless lineages to determine candidate genes before functional analysis. In (B), child (sub-categorical) processes are indented below parent processes. Categories with $P < 0.05$ are shown; full results are given in Table S11 [32]; 1284 total background genes were mapped to (A) 1229 IDs and (B) 1207 IDs; 21 candidate genes were mapped to 21 IDs (A and B).

Table 3: Genes detected to be under relaxed selection (higher dN/dS ratios) in flightless pterygote lineages as compared to related flying lineages as over-represented in (A) GO categories from DAVID analysis and (B) Biological Process categories from PANTHER analysis

(A) DAVID GO results					
GO term	1231 total genes # in category	54 higher dN/dS genes		P value	Fold enrichment
		Expected #	Observed #		
mRNA splicing, via spliceosome	50	2.2	8	0.0069	3.6
Catalytic step 2 spliceosome	35	1.5	6	0.021	3.9
Precatalytic spliceosome	39	1.7	6	0.033	3.5
(B) PANTHER Biological Process results					
PANTHER GO-Slim Biological Process term	1209 total genes # in category	53 higher dN/dS genes		P value	Fold enrichment
		Expected #	Observed #		
RNA localization	11	0.5	3	0.013	6.2
Death	13	0.6	3	0.020	5.3
Cell death	13	0.6	3	0.020	5.3
Apoptotic process	13	0.6	3	0.020	5.3
Negative regulation of apoptotic process	1	0.04	1	0.043	22.8
Localization	144	6.3	12	0.020	1.9
Extracellular transport	1	0.04	1	0.043	22.8

In (B), child (sub-categorical) processes are indented below parent processes. Categories with $P < 0.05$ are shown; full results are given in Table S11 [32]; 1285 total background genes were mapped to (A) 1231 IDs and (B) 1209 IDs; 56 candidate genes were mapped to (A) 54 IDs and (B) 53 IDs.

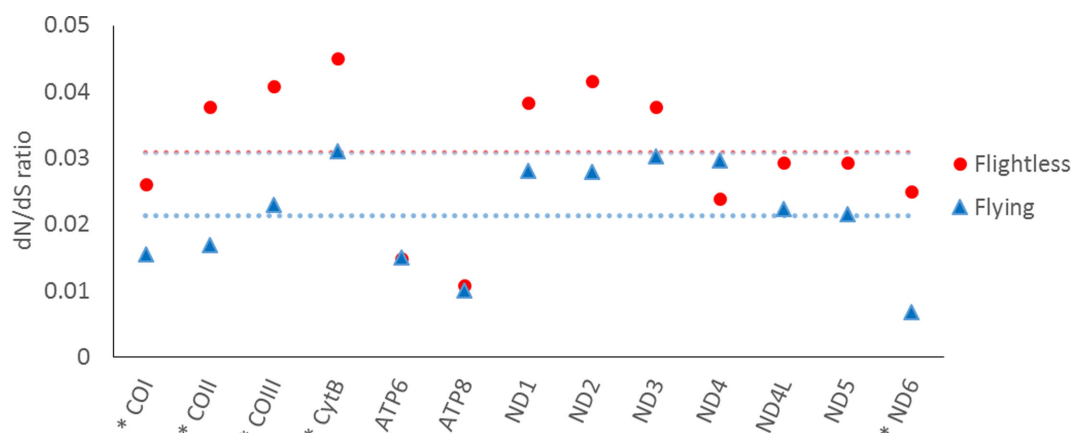


Figure 2: dN/dS ratios in flightless vs related flying lineages for 13 mitochondrial protein-coding genes. In 11 of 13 genes, the dN/dS ratio in the flightless pterygote lineages is higher than the dN/dS ratio of flying lineages. Genes with a significant difference in rates (after Benjamini-Hochberg correction) are marked with an asterisk; in all 5 cases, the dN/dS ratio is higher in the flightless lineages than in their flight-capable counterparts. Dashed lines signify the mean dN/dS values; flightless: 0.031 and flying: 0.021. The tree with lineages tested is provided in the S10 tree file [32].

Positive selection in nuclear and mitochondrial OXPHOS genes in hexapod lineages

Six of the 14 nuclear OXPHOS genes present in the total gene set exhibited positive selection in at least 1 branch (tree with 1 species chosen per order, represented in Fig. 3), along with 4 of 10 nuclear genes that were randomly selected to use as point of comparison, and 3 of the 5 other nuclear genes chosen *a priori* (genes listed in Table S12) [32]. Each mitochondrial OXPHOS gene had positive selection detected in at least 1 branch in either the 32-species tree with 1 species selected per order (Fig. 3) or the 66-species tree with multiple species selected per order (results in Table S13) [32]. The apterygote lineages (i.e., primarily flightless lineages, highlighted in grey) and lineages in orders Odonata (i.e., dragonflies and damselflies) and Ephemeroptera (i.e., mayflies), which have a direct flight mechanism, did not exhibit many signatures of positive selection, except in Protura and the interior branch leading to Protura + Collembola (Fig. 3). In the mitochondrial tree including more than 1 species per order, again no positive selection was detected in apterygotes (except in Protura), but some instances of positive selection were revealed within the Odonata + Ephemeroptera clade (Table S13) [32]. Positive selection in mitochondrial OXPHOS genes was more common in the holometabolous (i.e., complete metamorphosis) insect clade (labeled “H” in Fig. 3) than in the polyneopteran clade (labeled “L” in Fig. 3); both of those clades contain a similar number of orders and are of similar age (approximately 362 and 387 million years old, respectively) [2]. Nuclear genes showed little difference in the prevalence of positive selection between holometabolous and polyneopteran clades (8 vs 7 instances).

Discussion

This study tested for trends in the categories of genes evolving under differing selective pressures associated with flight evolution and loss in hexapods. The incorporation of both transition directions allows a comparison of trends in the genes under adaptive evolution and relaxed selective constraints with the evolution and loss of flight, respectively. We observed the origin of Pterygota to be associated with significant signatures of positive selection in categories of genes tied to catabolic processes and spliceosome, the latter overlapping with gene

categories represented by relaxed selection tests in lineages having undergone flight loss. Flight loss was also accompanied by positive selection in various categories of genes. These tests did not reveal any significant selection pressures in nuclear energy-related genes associated with flight evolution and loss, while mitochondrial genes displayed trends in line with previous expectations of relaxed selection associated with flight loss [9, 17, 18]. The holometabolous insects had the highest prevalence of positive selection.

OXPHOS genes related to flight: *a priori* gene selection

Energy-related genes, specifically mitochondrial and to a lesser extent nuclear OXPHOS genes, were expected to show signatures of positive selection with the origin of active flight and relaxed selection with the loss of flight. In a study of bat flight evolution [10], the lineage leading to bats was associated with 23% of mitochondrial-encoded OXPHOS genes displaying positive selection, while positive selection was only 3% more common in bat nuclear OXPHOS genes than in the lineage leading to rodents; other mitochondrial-associated nuclear genes showed no difference between lineages. In our study, no positive selection was observed associated with the origin of Pterygota for the mitochondrial OXPHOS genes, and no over-representation of positive selection was observed for nuclear OXPHOS genes as compared with the background gene sets or with other deep branches (“U” and “D”). It is possible that some signatures of selection were too difficult to detect due to the long time frames, given the trends in mitochondrial and nuclear OXPHOS genes in other insect [17], bird [9], and bat [10] taxa that have evolved or lost flight more recently. The origin of flight in Pterygota occurred approximately 400 million years ago, while bats originated about 60 million years ago [36].

Associated with flight loss, nuclear OXPHOS genes surprisingly more often showed higher dN/dS ratios in flying than flightless lineages (with 4 genes having significant differences), which was contrary to expectations when testing for relaxed selection. However, mitochondrial OXPHOS genes showed evidence of relaxed selection in flightless as compared with flying lineages, as demonstrated by significantly higher dN/dS ratios in flightless lineages. This is in accordance with previous observations of proposed relaxed selection in mitochondrial genes associated with flight loss within insect orders [17] and in birds

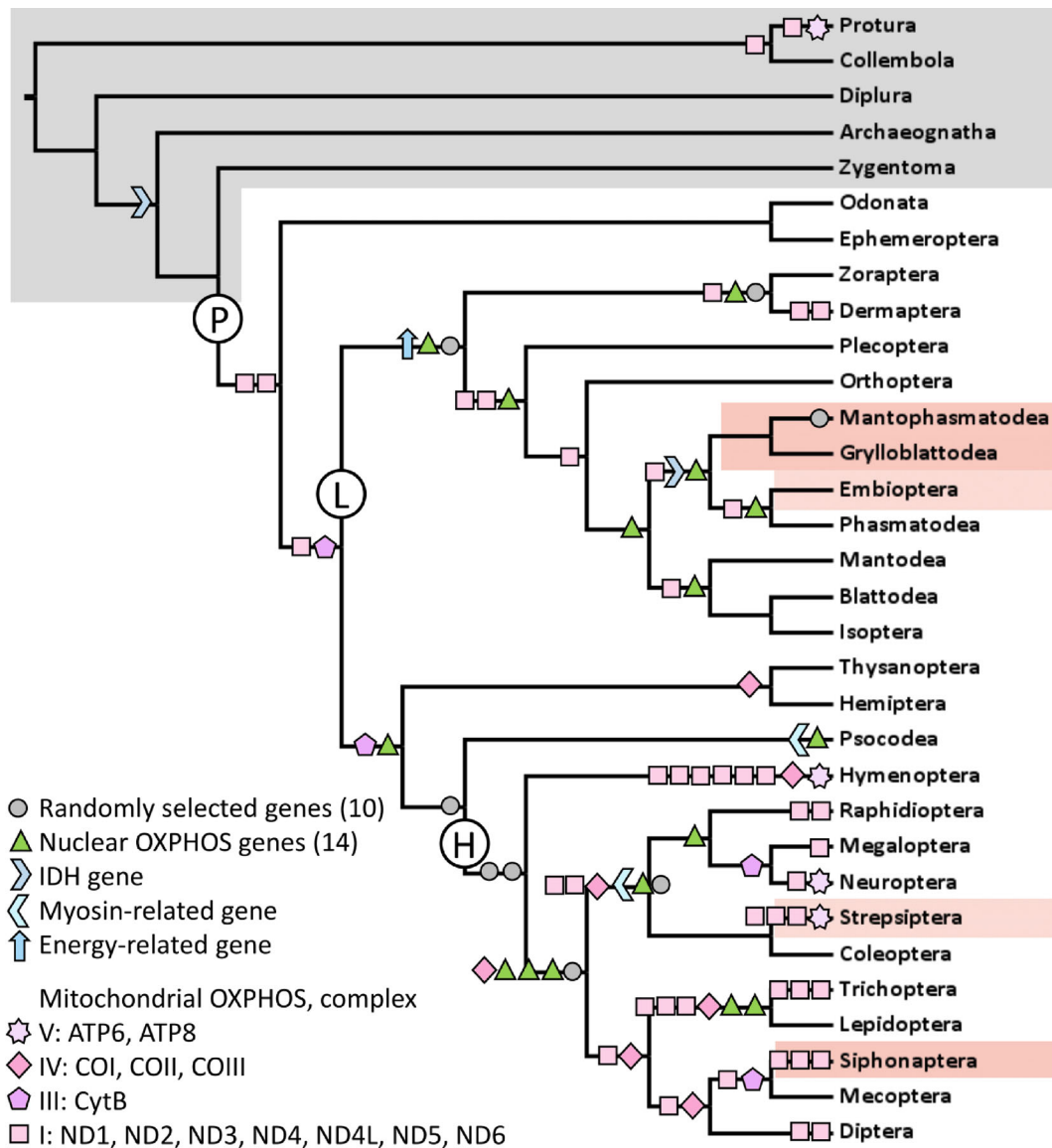


Figure 3: Positive selection in hexapod lineages in nuclear and mitochondrial genes of interest. The tree is adapted from Misof et al. [2], showing orders and involving 1 species representative per insect order for each gene tested. Orders/lineages that are shaded grey are apterygote (i.e., hexapods that never evolved the ability to fly), and those shaded orange consist entirely of species that are flightless due to a secondary loss of flight after its origin in Pterygota; note Embioptera and Strepsiptera are female flightless only. The lineage marked with "P" represents the lineage leading to the clade Pterygota; "L" = polyneoptera and "H" = holometabola (i.e., complete metamorphosis) insects.

[9]. These findings also mirror patterns of molecular evolution in weakly vs highly locomotive fish [37] and mammals [9]. Four out of the 5 significant differences in dN/dS ratios between flightless vs flying insect lineages were observed in the mitochondrial cytochrome genes (COI, COII, COIII, CytB), while only 1 significant difference was present for the other mitochondrial OXPHOS genes. These differences among genes could stem from varying levels of purifying selection. dN/dS ratios of mitochondrial protein-coding genes in mammals suggest the greatest purifying selection on sequences of COI, COII, COIII, and CytB [38], while in beetles the lowest rates of substitutions at first and second codon positions were observed in COI, CytB, ND1, COIII, and COII [39]. Thus, the trends between flightless vs flying lineages in their COI, COII, COIII, and CytB genes could be due to greater purifying selection on those genes in general, thus al-

lowing the effect of relaxed selection with flight loss to become apparent.

Previously, mitochondrial OXPHOS genes were examined for positive selection throughout a variety of phylogenetic lineages in insects, and there were fewer signatures of selection detected in apterygote lineages [18]. Here, we included all extant currently recognized insect orders, improving on the representation of both apterygote hexapod lineages (5 orders as compared to 2 included in Yang et al. [18]) and pterygote lineages (27 orders as compared to 20). We examined nuclear OXPHOS genes as well. We similarly observed a lack of positive selection in apterygote lineages, and nuclear OXPHOS genes were not disproportionately evolving under positive selection specifically associated with the origin of Pterygota. Mitochondrial OXPHOS genes exhibited substantial positive selection in the holometabolous

insects, while nuclear OXPHOS genes showed little proportional difference in comparison to the control genes. Although the number of taxa included here for holometabolous insects (clade “H” in Fig. 3) was similar to that for the polyneopteran clade (“L”), the holometabolous insects represent 83% of all insect species [40]. The detection of selection may in part be linked to the speciation rate of the group since species diversity and molecular evolutionary rates have been observed to correspond (e.g., [41]). However, this potential mechanism does not fully explain the findings as several highly species-rich groups (such as Lepidoptera) did not exhibit significant positive selection.

It was previously proposed that the type of flight mechanism—asynchronous vs synchronous flight—may explain trends in adaptive molecular evolution in flying insects [18]. Asynchronous flight, the ability for multiple wing beats per nerve impulse, is present for all of Hymenoptera (i.e., bees, wasps, ants, sawflies), Coleoptera (i.e., beetles), Strepsiptera (i.e., twisted-wing parasites), Diptera (i.e., flies), and Thysanoptera (i.e., thrips) [42]. However, these mechanisms may have similar energetic costs; although synchronous flight may cost more metabolically per stroke, asynchronous fliers often achieve higher stroke frequencies [43, 44]. The pattern of positive selection here does not mirror the occurrence of asynchronous vs synchronous flight. Positive selection associated with the origin of Pterygota was not greater than in downstream lineages. The origin of flight may have set the stage for downstream selection pressures within some lineages related to metabolic efficiency. However, other factors could also be influencing detection of positive selection in particular orders, such as fast mitochondrial gene substitution rates in Strepsiptera [45], proposed to be due to the transition to parasitism. The trend in holometabolous insects may, in general, relate to other biological traits tied to holometaboly itself, such as the occurrence of rapid development, which is thought to constrain genome size in that group [46]. Overall, the pterygotes have a greater prevalence of positive selection in OXPHOS (especially mitochondrial) genes than the apterygotes, as was expected tied to flight ability, with no apparent correspondence to any single flight-related mechanism.

Exploratory analysis of gene categories

In this exploratory analysis, we observed the origin of Pterygota to be associated with signatures of positive selection in protease and RNA catabolic processes genes, whose categories have a common theme of catabolism, which is the subset of metabolic activities involved in breaking down molecules to release energy and building components. Spliceosome-related genes were also over-represented in the positive selection results. The origin of Pterygota is associated with additional apomorphies other than flight, such as the evolution of metamorphosis and direct sperm transfer; as such, it is possible that results relate to functions other than flight or wings. The fit of GO categories with biological expectations would not validate the selection results as it is possible to create a biological narrative from inaccurate results through over-interpretation [47]. However, interestingly, the categories “proteasome” and “spliceosome” were also observed to be more highly expressed in flying vs flightless morphs of aphids [21].

Associated with flight loss, the gene categories exhibiting signatures of relaxed selection also frequently included “splicing” or “spliceosome.” The mirrored occurrence of this category between flight gain and loss suggests a biological association with flight in insects. While one transcription study has

linked expression levels of spliceosome-related genes to flying vs flightless morphs of cotton aphids [21], citrus and pea aphids do not exhibit a major difference in this category between flying and flightless morphs [22, 48], and expression differences in this category are only associated with sex-related differences within flying morphs in the brown planthopper [49]. “Localization” was also found to be a general category under relaxed selection in flightless insects, which mirrors the observation of over-representation of expression in the localization category between winged vs unwinged morphs of pea aphids [22].

Alternative splicing of exons in pre-mRNAs is one mechanism that contributes to increased phenotypic complexity [50], and as such, directional selection on splicing mechanisms may be congruent with the evolution of a complex trait such as flight ability. Alternative splicing is directly necessary for insect flight, which could account for splicing-related genes being under relaxed selection with flight loss as well. Almost all structural molecules in insect flight muscles, such as proteins and RNAs, exist as multiple isoforms [51]. Alternative splicing allows various isoforms of muscle-related molecules and as such appears to be an important mechanism to allow quantitative adjustment of muscle force and power output [51, 52]. However, it is unclear whether alternative splicing is more frequently occurring for these flight-related genes than all genes in general, as alternative splicing has been observed to occur in a large proportion of genes, at least in humans, including estimates of around 95% of multi-exon genes [53]. In addition, in multiple studies of flying vs flightless morphs of insects, there are no significant differences in expression levels of splicing genes, suggesting no large difference in general occurrence of splicing in flying vs flightless insects. However, flightless vs flying morphs of insects do not represent evolutionarily distinct lineages, and so genes exhibiting different expression levels among morphs may not be those bearing signatures of differences in selection regime between flightless vs flying insects on much longer evolutionary time scales. Thus, we suggest that genes related to splicing are a potential category for further investigation of whether differing selection pressures occurred with the origination of flight and flight loss in insects. This study examined only coding regions and was not able to consider changes in gene regulatory regions, which affect co-regulation. Co-regulation is important in processes including energy production [54]. Our results that suggest that alternative splicing as an important gene functional category for flight evolution may be a symptom of the involvement of regulatory changes in general, which we were not able to test here.

The loss of flight is not only associated with the change in flight ability, but also major changes in ecology and life history, such as diet, predation, habitat (e.g., woodlands, deserts), courtship, and often reduction in dispersal ability [8, 17]. Such changes are specific to certain species or clades, and thus the use of multiple lineages may help to eliminate some noise created by confounding biological or ecological factors. Nonetheless, some associated factors, such as reduced dispersal ability, are likely commonly associated with flight loss, and therefore, the results here are likely impacted by co-occurring factors in addition to change in flight capability itself. Categories of genes under positive selection associated with flight loss included protein motif (coiled coil), the nucleus, dendrite morphogenesis, and chromatin organization. These do not clearly fit with more highly expressed gene categories in flightless vs flying morphs of insect species observed by expression studies. For example, genes potentially undergoing positive selection with flight loss could be tied to sugar metabolism [48] or reproduction, such as

vitellogenin (an egg yolk protein precursor) [49]. Due to the generally reduced dispersal ability associated with flight loss and the energy trade-off between dispersal and reproduction [22], we expected positive selection in genes or processes tied to fecundity. For a gene to show signals of positive selection in multiple flight loss examples, the same ortholog must have adaptively diverged from flight-capable lineages. Given the long evolutionary history in the flight-adapted state before flight losses occurred and the seeming ease with which flight ability can be “turned off” developmentally by loss of function of specific genes [55], the relaxed selection tests may be more able to uncover trends in genes associated with flight loss than positive selection. Even so, given the consistent association between flight loss and increased reproductive ability, future studies using more genomic information may uncover positive selection with flight loss that we did not, or were not able to, detect here.

Caveats and next steps

The detection of positive selection can be affected by many factors including quality of the sequence alignment [56] and false positives and negatives associated with level of substitution saturation [57]. This study involved investigating positive and relaxed selection along longer time spans than are typical in genome-wide scan studies (e.g., approximately 60 million years separating dolphin vs cow) [58]. Thus, it is likely that positive or relaxed selection could be difficult to detect due to long time frames and various periods of positive and purifying selection, especially in the lineage leading to Pterygota. While GO categories are useful to look for trends in genomic selection, different gene categories could be detected under positive selection with varying species choice, change in background genes available, the Gene Ontology tool [59], or a version of the tool applied.

The replication provided by multiple losses of flight can help to narrow down uncertainty due to taxon selection and analysis methods, also helping to illuminate the interpretation of the molecular signatures associated with the single evolution of flight. Despite the long time frames included here, the trends observed for dN/dS ratios in flightless lineages as compared to flying lineages are similar to trends observed on shorter time frames within insect orders [17] and other animal taxa [9]. Future insect phylogenomic work with increased taxonomic sampling would allow further improvement in the number of cases of flight loss available, with increased accuracy of the phylogenetic mapping of transitions in flight state. Additionally, with better taxonomic sampling, the effects of co-occurring confounding factors (e.g., parasitism) could be separated, and trends for each type of flight loss (e.g., female flightlessness vs full flight loss) could be further investigated.

Importantly, expansion of the loci included in analysis would provide further insight into selection associated with flight gain and loss in insects. The single-copy, transcriptome-derived genes analyzed here represent a portion of all protein-coding genes in the insect genomes and thus restricted the total pool of possible gene categories that could be detected under differing selection pressures; for example, around 16,000 total genes are observed in *Drosophila* species [60]. Many gene functional categories are poorly represented in our data set, and thus the “expected” counts are low in some categories. The results of this study might therefore be considered hypotheses for testing using a larger portion of genomes in future studies. The orthologous genes included here represent those more essential for life as they are present and transcribed across a range of arthropod

species, life stages, and sexes; many serve basic cellular functions [2]. Thus, genes with more specialized functions, including some related to the development of wings or flying, are not represented. Furthermore, there may be important changes in regulatory (non-protein coding) regions, which govern expression levels and the specific tissues in which expression occurs, associated with flight and flight loss. Thus, future comparative genomics analysis using DNA-derived genomes could investigate both protein-coding and non-coding loci, as well as use full genomic data to assess gene gains or losses. Investigation of gene families would likely prove interesting, given that other studies have provided evidence for trends in adaptation based on gene presence and absence or gene family evolution, such as diversification among paralogous genes [60, 61].

Conclusions

This study presents an exploratory examination of the genes under positive and relaxed selection associated with the evolution and loss of flight in insects. Considering this study together with prior studies on other animal groups [9, 10, 17, 18], similarities were detected in the selection regime acting upon mitochondrial genes across multiple flying vs flightless animal groups. These results indicate convergent trends in molecular evolution that parallel convergent functional evolution in evolutionarily disparate animals. Various nuclear gene categories were linked to flight evolution and loss, which could be further explored for potential biological significance. Intriguingly, we found mirror-image patterns of selection in genes relating to splicing: positive selection with the origin of Pterygota and relaxed selection in flightless lineages. The results here contribute insight into the evolution of an important and unique trait that has played a major role in shaping the diversity of life.

Methods

Genetic data

Generation of the nuclear gene nucleotide alignments from the transcripts included these steps: (i) orthologous transcripts for each species were assigned to 1476 single-copy orthologous genes using an early version of Orthograph [62], version 0.5.4 (available from Github: <https://mptsrn.github.io/Orthograph/>); (ii) each gene was aligned with MAFFT v. 7.017 (MAFFT, [RRID:SCR_011811](https://doi.org/10.1093/molbev/msz119)) [63] using the L-INS-I algorithm for amino acid sequences translated from original nucleotide transcripts during orthology assignment; (iii) multiple sequence alignment of each orthologous gene was refined by identification of outlier sequences; refinement of outliers was performed using a profile alignment approach with MAFFT L-INS-I -add; the alignment was again checked for remaining outliers; final removal of outliers was performed; and (iv) a modified version (see [2]) of Pal2Nal [34] was applied to obtain the corresponding nucleotide multiple sequence alignments using the protein alignments as a blueprint.

Exploratory test of positive selection in lineage leading to Pterygota

Twenty-eight hexapod species were selected to maximize the number of shared nuclear genes available for analysis as well as the phylogenetic representation of pterygotes and non-ptyergote hexapods. Not all genes were available for all species in the candidate alignments, and thus species were selected

with the trade-off of number of species vs obtaining the largest gene set. For this test, we excluded flightless species or orders from within Pterygota, i.e., representing secondary flight losses. Species selection was performed in a phylogenetically stratified way, with the final list of 28 species being those that gave the maximum gene count: (i) all 5 apterygote orders were included, with a maximum of 3 species per order, but allowing up to 1 missing sequence per gene for this set; (ii) 1 species from Odonata and 1 species from Ephemeroptera were included, with no missing sequences allowed; (iii) 1 species per each of 5 orders of Polyneoptera was included, allowing 1 missing sequence per gene for this set; (iv) 1 species from each of 10 orders in the clade including Thysanoptera and Diptera (Fig. 1) was included, allowing up to 3 missing sequences per gene (species selected shown in Fig. 1). This resulted in 954 genes out of 1476. Similarly, 27 species representing apterygote and pterygote hexapod orders were selected for the 13 mitochondrial protein-coding genes, with no missing sequences allowed.

We tested for evidence of positive selection in these nuclear and mitochondrial genes in the lineage leading to Pterygota (Fig. 1, branch “P”). We used the branch site method of detecting positive selection [64] in the program PAML *codeml* version 4.8 (PAML, [RRID:SCR.014932](https://doi.org/10.1093/molbev/msw032)) [65], with the fit of models A1 (non-synonymous-to-synonymous [dN/dS] ratio fixed at 1) vs A (dN/dS ratio free to vary; each model with 4 classes of sites, each class allowing a certain combination of dN/dS ratios representing positive selection, purifying selection, or neutral evolution) compared for each gene separately through likelihood ratio tests [66]. For this and subsequent analyses, we corrected for false discovery due to multiple genes being tested by using the Benjamini-Hochberg correction [67] for each gene within a set, with a family-wise alpha of 0.05.

We repeated the tests on 2 additional lineages to serve as a null hypothesis to compare to the results for the lineage “P.” Branches “U” (upstream) and “D” (downstream) (Fig. 1) were tested. Using these results, we separated out genes that were uniquely detected as being under positive selection in the lineage leading to pterygote insects. These unique genes were subjected to GO analysis, described in the “Functional analysis” section below.

Exploring genes under positive selection with flight loss

Eleven cases of flight loss were identified by mapping flight state on the available phylogenetic tree (Fig. 1) adopted from Misof et al. [2], and 3 of these cases involved flight loss only in the female sex. Not all of these evolutionary losses were accurately mapped to the correct branch here, given the available species sampled. For example, a loss may have occurred in the common ancestor of a family, but only species representing superfamily-level divergences were available for our analysis. In the case of phasmids, flight loss occurred multiple times within the order [7, 68]. However, all available species were flightless, and thus the losses could not be represented accurately on the phylogeny; we tested the branch leading to the phasmid clade to approximate the timing of early flight losses in that order. Due to incomplete phylogenetic mapping of some of the flight loss events, the branches tested here likely represent some flying lineage history in addition to flightless lineage history, which may cause underestimation of molecular signal due to flight loss. A qualitative assessment is provided to indicate the likely degree of accuracy in the mapping of each case of flight loss, considering the density of taxonomic sampling in that group and how frequently flight is thought to have been lost in those groups (Fig. 1 and

Table S4) [32]. Sub-trees including the lineage of interest, sister lineage(s), and 3 successively branching outgroups were used to test for signatures of positive selection associated with each case of flight loss separately in order to maximize gene coverage; no missing gene data were allowed for the species within each sub-tree. Each sub-tree contained 14 to 19 species, with 584 to 1174 genes available for all species in each analysis (listed in Table S4) [32]. A total of 1284 genes were included, considering all 11 sub-trees.

A test for positive selection was performed on each of the 11 branches of interest for each sub-tree and gene separately. Those genes with significant P values (at 0.05 level after Benjamini-Hochberg correction) within a sub-tree were included in further analysis. We identified genes that were detected as evolving under positive selection in 3 or more of the 11 lineages tested. However, in order to eliminate those genes exhibiting a signature of selection in many lineages regardless of flight state, we also tested 9 flight-capable lineages that were sister lineages or were closely related to the flightless lineages for positive selection using the same sub-trees as the flightless lineages (trees and results in Mitterboeck et al. [32]). There were numerous flight loss events in 1 sub-tree, and so there were fewer related flight-capable lineages to include, resulting in 9 flight-capable lineages tested overall (as compared with 11 flightless lineages). The counts of genes exhibiting a significant signature of positive selection ($P < 0.05$ after Benjamini-Hochberg correction) were tallied for the flying lineages, and these counts were subtracted from the list of candidate genes for the flightless lineages. Those remaining genes with 3 or more counts of positive selection in the flightless lineages were included in functional analysis. This procedure was repeated for the 8 cases of full flight loss (i.e., excluding the 3 cases of female-only flight loss) as compared to 7 related flying lineages.

Exploring genes under relaxed selection with flight loss

Nuclear and mitochondrial genes were examined for relaxed selection associated with flight loss using branch models in PAML *codeml* to estimate dN/dS ratios for lineages of interest. For nuclear genes, the total 113-species tree (Fig. 1) was used, and missing data were allowed. Only genes with data for 80 or more species were included, resulting in 1285 genes tested. Flightless lineages representing full flight loss (not female-only flight loss) were coded 1 branch rate (red branches in Fig. 1), and the sister or related flight-capable lineages of similar tip number and taxonomic rank were coded together a separate rate (blue branches in Fig. 1), while all other lineages were coded as the background rate.

For each gene, a change in selection regime associated with loss of flight was concluded when there was a significantly increased dN/dS ratio (between 0 and 1) in flightless lineages as compared to flying lineages. Likelihood ratio tests between 3-rate trees (flightless [red], flying [blue], background [black + purple]) and 2-rate trees (flightless [red] + flying [blue] branches vs all other lineages [black + purple]) were used to test for significant dN/dS differences between target lineages and sister lineages. P values were corrected by Benjamini-Hochberg correction across genes, with a family-wise α of 0.05. Those genes that had a significantly higher dN/dS ratio in the flightless than flying lineages were examined by functional analysis (below) as compared to the total gene set tested. We interpreted increased dN/dS ratios as signifying relaxed selection. This interpretation of the dN/dS ratios involves the assumption that the majority of non-synonymous changes across a whole gene sequence

are selectively neutral or slightly deleterious; by contrast, positive selection is assumed to affect a small minority of sites at which mutations with beneficial effect have occurred [69]. However, given that increased dN/dS ratios can be due to strong positive selection rather than relaxed selection (or, in combination, in different parts of the gene), as a precaution we verified whether any genes from this list overlapped with those in the final candidate list for genes under positive selection in both sexes–flightless lineages.

For mitochondrial genes, a 66-species tree adopted from Misof et al. [2] (similar to Fig. 1) was used, given in the Mitterboeck et al. S10 tree file [32]. Since there is no “background” gene set due to all mitochondrial genes being energy related, we directly compared the dN/dS ratios in the flightless vs related flying lineages. In preliminary tests on these mitochondrial genes and in Mitterboeck and Adamowicz [17], the female-flightless lineages yielded similar results to full-flightless lineages as compared with related flying lineages. Due to this, and the smaller number of flightless lineages in the mitochondrial gene tree, we considered both female- and both sexes–flightless lineages in the flightless category (e.g., red + purple flightless lineages vs blue flying lineages, with the black lineages all coded to the background rate).

Functional analysis

We tested for over-representation in GO categories by the genes exhibiting positive or relaxed selection as compared to each total gene set analyzed (“background genes”) using the Database for Annotation, Visualization and Integrated Discovery version 6.8, October 2016 (DAVID, [RRID:SCR.003033](#)) Functional Annotation chart tool [70, 71] to identify enriched annotation terms and similarly using Protein Analysis Through Evolutionary Relationships version 11.1 (PANTHER, [RRID:SCR.004869](#)) [72] to identify “processes.” The genes were matched to *Drosophila* genome functional annotations where available, using the FlyBase ID [2, 73] for each gene. No additional false discovery rate correction was applied (*P* values are raw) as correction was already applied for the positive selection analysis, and also the number of candidate genes was lower than the optimal working input for DAVID (hundreds to thousands of genes) [70]. For DAVID analysis, the expected number of genes in each GO category was calculated by the number of genes in the category divided by the number of background genes, multiplied by the number of candidate genes (e.g., expected number in Table 1 first GO term; $(34/914) \times 38 = 1.4$). The fold enrichment was calculated by the observed number divided by the expected number of genes in that GO term. The 1476 source genes used in our analysis are not representative of gene categories in the full insect genomes; we provide information on the gene categories over- or under-represented by these 1476 genes in relation to the full genome of *Drosophila melanogaster* in Table S14 [32]. Our tests for over-representation of genes under positive or relaxed selection are in relation to each of our available gene sets, i.e., “background” sets that are each a subset of the total 1476 genes. In addition to GO analysis, which provides information on functional terms based on over-representation available in the candidate gene set, we grouped lists of genes in similar DAVID terms (those terms present with default settings in the “chart” or “cluster” mode) existing in our background 1476 gene set. The grouped terms were those related to each (i) wing development (4 chart terms and 1 cluster term for a total of 48 genes) and (ii) mitochondrion/ATP-binding/respiratory-chain-related functions (7 chart and 11 cluster terms for a total of 237 genes). We acknowledge that these

groupings do not include all possible genes related to the wing or mitochondrion-related functions of interest in the data set but provide 2 larger, functionally defined groupings to test. Grouping similar GO terms for analysis can improve interpretation of results by increasing statistical power that is diminished by the dependence between GO terms, thus revealing trends not detected in individual GO terms [74]. We test for over- and under-representation of these gene sets in candidate vs non-candidate lists using 1-tailed Fisher’s exact tests in R version 3.3.1 (Table S16) [32, 75]. Also, we report whether nuclear OXPHOS genes were over- or under-represented in the candidate gene sets, with genes identified through names provided in Tripoli et al. [14].

Positive selection in energy-related genes in Hexapoda

Specific genes were investigated that related to energy production or were *a priori* hypothesized to be related to flying or flight loss. These included 14 nuclear OXPHOS genes available in the total gene set (1476 genes) identified via their FlyBase IDs, which are a subset of the 78 nuclear OXPHOS genes listed in Tripoli et al. [14], and 5 other genes of interest identified by name or description in DAVID functional annotation: *wingless*, *IDH*, *flightless1*, *myosin binding subunit*, and an energy-related gene (*Dmel.CG1271*). Ten additional genes with full species coverage were pseudo-randomly selected (not considering function, with the selections spread out by FlyBase IDs) and also analyzed to check for phylogenetic biases in the positive selection results. We selected 1 species per hexapod order (32 orders) and 1 species from each of 2 arthropod outgroups (outgroups were available for the nuclear genes only; 34 species total) for each set of nuclear and mitochondrial genes. In selecting species, we considered gene completeness, with preference for those species available across the most genes of interest. In a few cases, substitutions of some species were made to improve gene completeness (species lists provided in Table S12) [32]. Mitochondrial genes, where gene sampling was more complete for species, were additionally tested with more than 1 species per order (up to 6 species) to investigate the effects of species sampling on the results; the 66-species tree was the same as that used for relaxed selection analysis of mitochondrial genes (in Table S10) [32]. Tests for positive selection were conducted on all lineages using the program HyPhy [76] and the branch site Random Effects Likelihood model [77] implemented on the publically available DataMonkey server (Data Monkey, [RRID:SCR.010278](#)) [78].

Availability of data and materials

The data sets supporting the results of this article are available in the GigaDB repository associated with this publication [32], including input and output information such as gene lists, newick trees, *P* values for selection tests, and functional analysis results. The nuclear sequence data are available associated with the NCBI Project PRJNA183205.

Abbreviations

GO: gene ontology; PAML: Phylogenetic Analysis by Maximum Likelihood.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the University of Guelph (Integrative Biology PhD Award, Dean's Tri-council Scholarship, and Ontario Graduate Fellowship to T.F.M.), the Government of Ontario (Ontario Graduate Fellowship to T.F.M.), and the Natural Sciences and Engineering Research Council of Canada (Alexander Graham Bell Canada Graduate Scholarship to T.F.M., Discovery Grants 386 591–2010 to S.J.A. and 400 479 to J.F.). X.Z. is supported by the China Agricultural University through the Chinese Universities Scientific Fund (2017QC114).

Authors' contributions

Conceived or designed work: X.Z., T.F.M., R.Z., W.S., J.F., S.J.A., S.L. Filtered genetic data: K.M., S.L. Designed data sets and analyses: T.F.M., S.L. Conducted bioinformatics for PAML analyses: S.L. Conducted gene ontology and HyPhy analyses: T.F.M. Drafted the article, generated figures and tables: T.F.M. Revised article drafts: T.F.M., S.J.A., S.L., J.F., K.M. All authors have read and approved the final manuscript.

Acknowledgements

We thank Lili Zhou for her contribution during the early stages of this project. We give a huge thank you to the 1KITE community, who have made the data possible, especially Alexander Donath and Lars Podsiadlowski, who worked on the current transcriptome assembly, cross-contamination check, and submission to NCBI. We thank Stephen Marshall and Daniel Ashlock for input on the ideas in earlier versions of this manuscript and Jim Marden and Christopher Jones for valuable input on the manuscript as reviewers.

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